

ORIGINAL ARTICLE

Genetic divergence, range expansion and possible homoploid hybrid speciation among pine species in Northeast China

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Although homoploid hybrid speciation in plants is probably more common than previously realized, there are few well-documented cases of homoploid hybrid origin in conifers. We examined genetic divergence between two currently widespread pines in Northeast China, *Pinus sylvestris* var. *mongolica* and *Pinus densiflora*, and also whether two narrowly distributed pines in the same region, *Pinus funebris* and *Pinus takahasii*, might have originated from the two widespread species by homoploid hybrid speciation. Our results, based on population genetic analysis of chloroplast (cp), mitochondrial (mt) DNA, and nuclear gene sequence variation, showed that the two widespread species were divergent for both cp- and mtDNA variation, and also for haplotype variation at two of eight nuclear gene loci surveyed. Our analysis further indicated that *P. sylvestris* var. *mongolica* and *P. densiflora* remained allopatric during the most severe Quaternary glacial period that occurred in Northeast China, but subsequently exhibited rapid range expansions. *P. funebris* and *P. takahasii*, were found to contain a mixture of chlorotypes and nuclear haplotypes that distinguish *P. sylvestris* var. *mongolica* and *P. densiflora*, in support of the hypothesis that they possibly originated via homoploid hybrid speciation following secondary contact and hybridization between *P. sylvestris* var. *mongolica* and *P. densiflora*.

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INTRODUCTION

Hybridization is widespread in plants and has an important role in plant diversification and evolution (Abbott, 1992; Rieseberg *et al.*, 2003; Arnold, 2006). Hybridization occurs in situations of sympatry and parapatry, often following range shifts and secondary contact between closely related and formerly allopatric species (Song *et al.*, 2002, 2003; Wachowiak and Prus-Głowacki, 2008). Hybridization can result in introgression of alleles across species boundaries and sometimes the origin of a new introgressant lineage (Currat *et al.*, 2008; Kim *et al.*, 2008). Additionally, it can trigger the origin of a new hybrid species through allopolyploid or homoploid hybrid speciation (Rieseberg, 1997; Mallet, 2007; Abbott *et al.*, 2010). Whereas allopolyploid speciation is common in plants, homoploid hybrid speciation is regarded as rare (Rieseberg, 1997; Gross and Rieseberg, 2005), although more examples of homoploid hybrid species are likely to be discovered in the future now that genetic resources are readily available for testing their occurrence.

In contrast to allopolyploid speciation, which is relatively easy to identify from chromosomal and molecular data, homoploid hybrid speciation is difficult to detect unambiguously (Rieseberg, 1997; Mallet, 2007). Nevertheless, a small but increasing number of cases has been documented recently across a range of plant, animal and fungal taxa (Gross and Rieseberg, 2005; Mallet, 2007; Abbott *et al.*, 2010). It is well known that many closely related pine species

hybridize, producing fertile and vigorous hybrids that combine morphological traits and/or genetic components of both parental species (Mirov, 1967; Wachowiak and Prus-Głowacki, 2008). Although several Asian pine species are thought to be of hybrid origin (Mirov, 1967), only *Pinus densata* has been rigorously analyzed and shown to be the homoploid hybrid of *Pinus tabulaeformis* and *Pinus yunnanensis* (Wang *et al.*, 1990; Song *et al.*, 2002, 2003, 2011; Ma *et al.*, 2006).

Here, we investigate mitochondrial (mt), chloroplast (cp) and nuclear DNA variation at the population level within four closely related pine species in Northeast China that have the same chromosome number ($2n=24$; Zhang and Li, 1984). Two of these species, *Pinus sylvestris* var. *mongolica* and *Pinus densiflora*, are dominant components of natural coniferous forests in Northeast China (Wu, 1995), a region considered not to have been glaciated during the Quaternary (Hewitt, 2000). However, climatic oscillations during the Quaternary may have caused these indigenous coniferous forests to retreat to refugia during glacial periods and undergo range expansions during interglacials (Wu, 1995; Aizawa *et al.*, 2007; Chen *et al.*, 2008) as recorded for forest trees in other regions (for example, Petit *et al.*, 2003; Naydenov *et al.*, 2007). Such range shifts can stimulate allopatric divergence during periods of range fragmentation across different glacial refugia (Hewitt, 2004), but provide opportunities for interspecific hybridization following contact during interglacials, although

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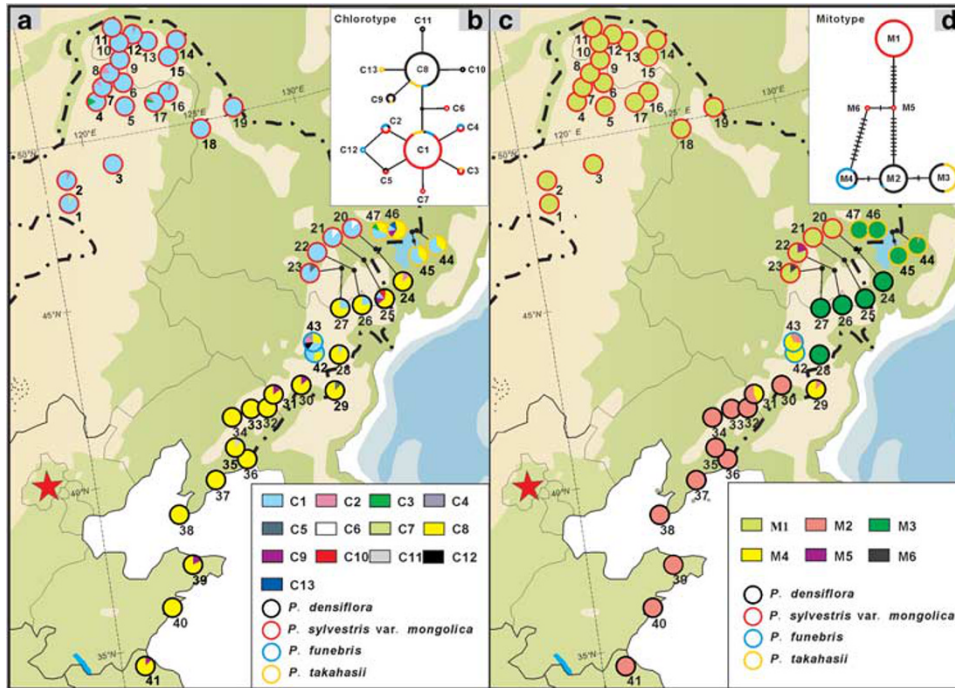


Figure 1 Distributions and networks of chlorotypes (a, c) and mitotypes (b, d) among four pine species in Northeast China. The color of the circumference of circles in (a) and (c) indicates each species and in (b) and (d) is proportional to the frequency of each chlorotype or mitotype in each species. The color filled in circles in (a) and (c) is proportional to the frequency of each chlorotype or mitotype in each population.

the possibility of subsequent homoploid hybrid speciation has rarely been examined or reported (Abbott and Brochmann, 2003). *P. sylvestris* occurs widely from Europe to Eastern Asia with var. *mongolica* being widely distributed in mountainous areas of the northern part of Northeastern China. In contrast, *P. densiflora* occurs only in Northeast China, Korea and Japan, being found more to the south in Northeastern China and in the Shandong Peninsula (Cheng and Fu, 1978) (Figure 1). A phylogenetic analysis based on four cpDNA markers, previously indicated that *P. sylvestris* and *P. densiflora* comprise a pair of sister species within a small pine monophyletic lineage (Wang *et al.*, 1999). They are distinguished by several morphological traits, including differences in needle tips and number of resin canals (Table 1). Phylogeographic analysis of *P. sylvestris* suggests that the East Asian populations became established following post-glacial range expansion of the species from glacial refugia in central Asia (Naydenov *et al.*, 2007).

The two other pine species investigated here, *Pinus funebris* and *Pinus takahasii*, have very narrow distributions and might potentially have originated by homoploid hybrid speciation following hybridization between *P. sylvestris* var. *mongolica* and *P. densiflora* (Cheng and Fu, 1978). *P. funebris* occurs in a restricted region of the eastern Changbai Mountains where it grows at elevations between 800 and 1600 m, partly overlapping with *P. densiflora*, which occurs below 900 m. In contrast, *P. takahasii*, occurs allopatrically around the Xingkai Lake in moist habitats. There is general agreement that both *P. funebris* and *P. takahasii* are intermediate to *P. sylvestris* var. *mongolica* and *P. densiflora* in some morphological traits (Table 1; Takenouchi, 1942; Mirov, 1967; Cheng and Fu, 1978; Fu *et al.*, 1999). In the past, some investigators have placed them as different forms of either *P. densiflora* (for example, Takenouchi, 1942) or *P. sylvestris* (Cheng and Fu, 1978) rather than considering them as distinct species. However, both are phenetically and ecologically different from

P. sylvestris var. *mongolica* and *P. densiflora* (Takenouchi, 1942; Cheng and Fu, 1978; Fu *et al.*, 1999) and have been treated as distinct species by some taxonomists. Szmidi and Wang (1993), although not treating *P. funebris* as a distinct species, previously showed that plants of this type contained a mixture of allozyme polymorphisms from *P. sylvestris* var. *mongolica* and *P. densiflora*.

Pinus species exhibit paternal cp inheritance transmitted via pollen and maternal mt inheritance via seeds (Song *et al.*, 2003; Chen *et al.*, 2008; Zhou *et al.*, 2010). This independent inheritance of two cytoplasmic genomes provides the opportunity for tracing paternal and maternal genetic changes within a single species because of different rates of gene flow and for discriminating female and male parents of hybrids (Ennos, 1994; Liston *et al.*, 2007; Wachowiak and Prus-Głowacki, 2008). In addition, the analysis of multiple nuclear loci can be used to examine interspecific divergence and clarify parental origins of putative hybrid species (for example, Ma *et al.*, 2006; Li *et al.*, 2010). Thus, here we use sequences from cpDNA, mtDNA and eight nuclear genes to address the following specific questions. (1) Are *P. sylvestris* var. *mongolica* and *P. densiflora* clearly divergent for cp-, mt- and nuclear DNA? (2) How did these two widely distributed species in Northeast China respond to Quaternary climatic oscillations in terms of range shifts? (3) Is it possible that diploid *P. funebris* and *P. takahasii* originated following interspecific hybridization between *P. sylvestris* var. *mongolica* and *P. densiflora*?

MATERIALS AND METHODS

Population sampling

Needles were collected from 489 individuals across 47 natural populations throughout the natural distribution ranges of *P. sylvestris* var. *mongolica*, *P. densiflora*, *P. funebris* and *P. takahasii* (Figure 1, Supplementary Table S1). At four sites, *P. sylvestris* var. *mongolica* and *P. densiflora* occurred sympatrically (that is, at sites where populations 20–23 of *P. sylvestris* var. *mongolica* and

Table 1 Morphological variation between *P. sylvestris* var. *mongolica*, *P. densiflora*, *P. funebris* and *P. takahashii* (from Cheng and Fu, 1978; Fu *et al.*, 1999)

Character	Parental species		Assumed hybrid species	
	<i>P. sylvestris</i> var. <i>mongolica</i>	<i>P. densiflora</i>	<i>P. funebris</i>	<i>P. takahashii</i>
Bark color	Yellow, brown-yellow	Orange-red, red-brown	Brown-yellow, golden yellow	Red-brown, brown-yellow
First-year branchlets	Not white powdery	White powdery	Not white powdery	White powdery
Winter buds	Brown or pale yellowish brown	Dark red-brown	Red-brown	Red-brown
Number of needles	2	2	2	2
Length of needles (cm)	4–9	5–12	5–8	5–10
Needle diameter (mm)	1.5–2	1	1–1.5	1–1.5
Needles twisted or not	Yes	No	No	Yes
Number of resin canals	6–11	4–6	4–8	8
Seed cones	Pendulous	Erect	Pendulous	Pendulous
Cone length (cm)	3–6	3–5.5	4–5	4–5
Cone diameter (cm)	2–3	2.5–4.5	3–4.5	2–3
Seed and wing length (cm)	1.1–1.5	1.5–2	2	1.5
Florescence	May–June	April	May–June	May–June
Timber	Flexible	Brittle	–	–

populations 24–27 of *P. densiflora* co-occurred). Populations of the two possible hybrid species occurred allopatrically in the eastern or western Changbai Mountains and because of their restricted distributions only two and four populations of *P. funebris* and *P. takahashii* were sampled, respectively. In all, 7 to 12 individuals were sampled in each population, making sure that all individuals sampled were at least 100 m apart. We further used seeds from 78 trees of 18 populations (four to six individuals sampled per population, Supplementary Table S1) for sequencing nuclear loci. The latitude, longitude and altitude of each location sampled were measured by Extrex GIS (German, Taiwan). All needle samples were dried and stored in silica gel after collection. Seeds were stored at -20°C , and soaked overnight in water at room temperature, before isolation of the haploid megagametophyte (the maternal tissue surrounding the embryo in gymnosperm seeds).

DNA extraction, amplification and sequencing

Total genomic DNA was isolated from 255 *P. sylvestris* var. *mongolica*, 148 *P. densiflora*, 37 *P. funebris* and 49 *P. takahashii* individuals, using approximately 20 mg of silica-gel dried, leaf-needle material per sample according to a hexadecyltrimethyl ammonium bromide procedure (Doyle and Doyle, 1987) modified for use with an electric tissue homogenizer (QIAGEN, manufactured by Retsch, Qiagen, Valencia, CA, USA). Similarly, megagametophytes of seeds were separated and total DNA was extracted for sequencing nuclear loci. These megagametophytes were regarded as random gamete samples from each population. A total of 78 haploid DNA genomes from megagametophytes were analyzed for sequencing nuclear loci.

Three cpDNA regions: *rpl16F71-rpl16R15*, *trnS-trnG* and *rbcl*, were amplified and sequenced following the methods described in Zhou *et al.* (2010). For mtDNA, the *nad4/3–4*, *nad5* intron 1 (Chen *et al.*, 2008), *nad1* intron B/C and *nad7* intron 1 (Naydenov *et al.*, 2007) regions that were found to be polymorphic in *P. tabulaeformis* and *P. sylvestris*, were surveyed for variation. Finally, for nuclear DNA we initially screened 20 loci across the four species and selected for further analysis only those loci represented by a single band generated by polymerase chain reaction (PCR). To check that such bands comprised a single sequence each PCR product was cloned into a pGEM T-easy vector (Promega Inc., Madison, WI, USA) and sequenced. Ten clones were sequenced per band. From the original 20 loci, eight (*a3ip2*, *coaomt*, *pod*, *c3h-1*, *CesA2*, *dhn1*, *erd3* and *aqua-MIP*) were selected for inclusion in the total survey. PCR primers and the putative function of these eight loci are listed in Supplementary Table S2. All PCRs were conducted in a 25- μl volume, containing 10–40 ng plant DNA, 50 mM Tris-HCl, 1.5 mM MgCl_2 , 250 $\mu\text{g ml}^{-1}$ bovine serum albumin, 0.5 mM dNTPs, 2 μM of each primer and 0.75 U of *Taq*

polymerase. PCR products were purified using a TIANquick Midi Purification Kit following the recommended protocol (TIANGEN, Beijing, China).

To obtain the complete nucleotide sequence of the *nad1* and *rbcl* regions, two to four different sequencing reactions were required using the PCR primers listed plus a pair of internal primers (*nad1*F1: 5'-TATTTCATAGGCAGCA GGTC-3', *nad1*R1: 5'-GGGTCTTGAGCGAACTACTC-3') designed from the known sequence in *P. sylvestris* (Naydenov *et al.*, 2007). These reactions were conducted on eight individuals per species, and then based on the complete sequences, two sequencing primers (*nad1*-R2: 5'-TCGCCCGACCTA AGAAGG-3' and *rbcl*-F1: 5'-AGGCTGAGACGGGTGA-3') were designed to sequence other trees included in the survey. All sequencing reactions used the ABI PRISM BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). DNA sequences were aligned with Clustal X and double checked by eye. All haplotype sequences are available from GenBank (accessions JF701446–JF701600).

Population genetic analysis

We used NETWORK version 4.2.0.1 to construct phylogenetic relationships among mtDNA, cpDNA and nuclear haplotypes (Bandelt *et al.*, 1999; available at <http://www.fluxus-engineering.com>). Average mtDNA and cpDNA gene diversity within populations (H_S), total gene diversity (H_T), and the coefficients of differentiation G_{ST} and N_{ST} were estimated for each species except *P. funebris*, which comprised less than three populations, using PERMUT (available at <http://www.pierroton.inra.fr/genetics/labo/Software/Permut/>). We also compared the two estimates of population divergence, G_{ST} (coefficient of genetic variation over all populations; Nei, 1973) and N_{ST} (coefficient of genetic variation influenced by both haplotype frequencies and genetic distances between haplotypes), using a permutation test with 1000 permutations. We further examined Hierarchical partitioning of diversity among species, populations and individuals by analysis of molecular variance using ARLEQUIN version 3.0 (Excoffier *et al.*, 2005), with significance tests based on 1000 permutations. As hybridization can result in recombination between some chlorotypes or mitotypes (Jaramillo-Correa and Bousquet, 2005), we used the four-gametic criterion defined by Hudson and Kaplan (1985) to infer possible recombination of cpDNA and mtDNA haplotypes. In doing this, we compared every pair of polymorphic nucleotide sites, for all four possible combinations of alleles (that is, Ab, AB, aB and ab) between the AB and ab haplotypes observed.

For nuclear sequences, we used DnaSP version 5.00.04 (Librado and Rozas, 2009) to estimate the number of segregating sites (S); Watterson's parameter (θ_w) (Watterson, 1975); nucleotide diversity (π) (Tajima, 1983), the minimum number of recombinant events (R_m) (Hudson and Kaplan, 1985) and net

sequence divergence (D_a) (Nei, 1987). We also calculated Tajima's D (Tajima, 1989), Fu and Li's D^* and F^* statistics (Fu and Li, 1993) and Fay and Wu's H (Fay and Wu, 2000) using the DnaSP version 5.00.04 software (Librado and Rozas, 2009). We used *P. ponderosa*, *P. taeda*, *P. radiata* and *P. koraiensis* as outgroups for Fay and Wu's H neutrality tests. In addition, Wright's fixation index, F_{ST} (Wright, 1951) was calculated for each locus and tested for significance by 1000 permutations as implemented in ARLEQUIN version 3.0 (Excoffier *et al.*, 2005). Indels were excluded from all calculations other than for F_{ST} .

We also used STRUCTURE V. 2.3 (Hubisz *et al.*, 2009) to assess the correspondence between species grouping and nuclear genotypic clustering. We assessed the likelihood of each number of clusters, K , ($1 \leq K \leq 10$) with allowance made for the correlation of allele frequencies between clusters. We performed 10 runs with a burn-in of 100 000 and then 500 000 iterations. We used Distruct v.1.1 to produce graphics (Rosenberg, 2004) and estimated the most likely number of clusters using the original method from Pritchard *et al.* (2000), and the ΔK statistics given in Evanno *et al.* (2005).

We used LAMARC v2.1.5 a coalescent-based method to estimate migrate rates (M) among the four species based on eight nuclear loci (Kuhner, 2006). This analysis used the Bayesian method with replication of chains and adaptive heating (Metropolis-Coupled Markov Chain Monte Carlo). Chains were repeated with different initial genealogies with each chain split into multiple searches, allowing for better sampling of parameter space. We used three independent MCMC runs of varying length and burn-in. All MCMC runs produced similar results, and therefore only results for the longest runs were presented, which were composed of three replicates of 10 initial chains and 2 long final chains. The initial chains were performed with 5000 samples and a sampling interval of 30 (150 000 steps), using a burn-in of 10 000 samples for each chain. The two final chains were carried out with the same burn-in and interval sampling, but with 50 000 samples (1 500 000 steps).

Tests for rapid expansion based on cpDNA sequences

Mismatch distributions of the observed number of nucleotide differences between pairs of cpDNA sequences were computed using the program Arlequin version 3.0. (Excoffier *et al.*, 2005) to detect historical expansions in *P. sylvestris* var. *mongolica* and *P. densiflora*, for which 'star-like' phylogenies of rare chlorotypes were exhibited. We assumed that populations at demographic equilibrium would have a multimodal distribution of pairwise differences, while those that experienced a sudden demographic expansion should display a unimodal distribution (Slatkin and Hudson, 1991). We tested three data sets: (I) all samples of *P. sylvestris* var. *mongolica*, (II) all samples of *P. densiflora* and (III) samples with chlorotypes specific to *P. densiflora*. We used a total of 1000 parametric bootstrap replicates based on segregating sites to generate an expected distribution under a model of sudden demographic expansion (Rogers and Harpending, 1992). We also used the sum of squared deviations (SSD) as a statistic to test the validity of the expansion model with P -values calculated as the proportion of simulations producing a larger SSD than the observed SSD. We calculated the raggedness index and its significance to quantify the smoothness of the observed mismatch distribution.

For each of the same three groups (I–III), we also calculated Fu's F_S value, which is primarily based on differences between expected numbers of alleles (estimated through 10 000 computer simulations based on the observed pairwise differences in our sample) and observed numbers of alleles. This statistic is very sensitive to a recent demographic expansion for which large, negative values are typically obtained (Fu, 1997). Values of Tajima's D (Tajima, 1989) were also calculated to assess population expansion (using the total number of mutations), for which negative values are also expected. Estimation and testing were conducted using Arlequin version 3.0 (Excoffier *et al.*, 2005) with 1000 bootstrap replicates for both Fu's F_S and Tajima's D .

To further assess the demographic history of species, we also used LAMARC v2.1.5 (Kuhner, 2006), which takes account of genealogical relationships among haplotypes, to estimate the exponential population growth rate parameter g . For the three groups (I–III), we also conducted three independent MCMC runs as detailed above. All MCMC runs produced similar results. Large and positive values of the exponential growth parameter g indicate population expansion, whereas negative values indicate population shrinkage. The scale of g is known

to be biased upward, and thus relatively small positive values ($g=10$) may indicate little or no growth, whereas small negative values ($g=-10$) may indicate important population size declines (LAMARC documentation: <http://evolution.gs.washington.edu/lamarcl/>).

Tests of alternative scenarios for the origins of *P. funebris* and *P. takahasii* by ABC modeling

To obtain a more detailed inference on the evolutionary histories of these four species, we tested potential scenarios using the approximate Bayesian computation procedure (ABC) in DIYABC v1.0.4.39 (Cornuet *et al.*, 2008, 2010) based on eight nuclear loci. Data can be simulated under any number of scenarios of population divergence, population size change and admixture (Cornuet *et al.*, 2008). We tested three possible origin scenarios of *P. funebris* and *P. takahasii*, respectively: admixture from both putative parental species, divergence only from *P. sylvestris* var. *mongolica* and divergence only from *P. densiflora*. Based on the cpDNA analysis, both *P. sylvestris* var. *mongolica* and *P. densiflora* experienced rapid range expansions. So, we added population size change models to these three scenarios (Supplementary Figure S1). For the ABC analysis, parameter values were set from the minimum to maximum range of priors. We chose the number of haplotypes, number of segregating sites and mean pairwise difference as one-sample summary statistics, and the mean of pairwise differences (W) and (B) as two-sample summary statistics to compare observed and simulated data sets. A reference table consisting of 1 000 000 simulated data sets per scenario was created. We used 1% of the simulated data sets closest to the observed data to estimate the relative posterior probability (with 95% confidence intervals (CIs)) of each scenario via a logistic regression and posterior parameter distributions according to the most likely scenario (Cornuet *et al.*, 2008, 2010). We assumed a generation time of 25 years for each of the four species as suggested for other pine species (Brown *et al.*, 2004) and our field observations.

RESULTS

cpDNA variation

Based on sequence variation exhibited in the three cpDNA fragments (*rpl16*, *trnS-trnG*, *rbcL*) analysed, 13 chlorotypes (Supplementary Table S3) were identified across all individuals of the four species sampled with 2 chlorotypes recorded as frequent (C1 and C8) and 11 as relatively rare (C2–C7, C9–C13) (Figure 1a; Supplementary Table S1). Whereas *P. sylvestris* var. *mongolica* possessed chlorotype C1 at high frequency (94.5%), *P. densiflora* was characterized by a high frequency of chlorotype C8 (88.5%). Chlorotype C1 was fixed in 12 of 23 populations surveyed in *P. sylvestris* var. *mongolica* and occurred at high frequency in the remaining populations. In the same species, chlorotypes C2–C4 and C7 were present at low frequency in north-western populations, while C5 and C6 occurred only in eastern populations that were sympatric with *P. densiflora*. Three of the chlorotypes found in *P. sylvestris* var. *mongolica* were also recorded at very low frequency in *P. densiflora*, that is, C1 in populations 25–28, C2 in population 24, and C5 in population 29. In *P. densiflora*, 7 of the 18 populations surveyed were fixed for chlorotype C8, while the remaining populations contained C8 at high frequency. Some populations of *P. densiflora* also contained at low frequency chlorotypes C9–C11, which along with C8 were never recorded in *P. sylvestris*. The two putative hybrid species, *P. funebris* and *P. takahasii*, contained a mixture of chlorotypes from both parent species. Thus, chlorotypes C1, C2, C4 and C8 were present in *P. funebris*, while C1, C3, C8 and C9 occurred in *P. takahasii*. In addition, each putative hybrid species contained one species-specific chlorotype at low frequency—C12 being specific to *P. funebris* and C13 being specific to *P. takahasii*.

The minimum-spanning network for chlorotypes (Figure 1b) identified two distinct groups, which mostly represent chlorotypes possessed by the two parental species, respectively. The putative hybrid species possessed chlorotypes from both groups. Values for average

within-population cpDNA diversity (0.535) and total genetic diversity (0.607) in *P. takahasii* were much higher than in *P. sylvestris* var. *mongolica* (0.109, 0.109) and *P. densiflora* (0.196, 0.201). N_{ST} and G_{ST} were low in both *P. densiflora* and *P. sylvestris* var. *mongolica*, and in neither case was N_{ST} significantly larger than G_{ST} (Supplementary Table S4). Analysis of molecular variance showed that (77.08%) of the total cpDNA variation was distributed between species, and that variation within species, was mostly due to that present within populations. Analysis of molecular variance conducted only on the putative parental species showed that 91.20% of the variation was distributed between them (Supplementary Table S5).

mtDNA variation

A combined analysis of mutations and indels across the four mtDNA fragments surveyed (*nad4/3-4*, *nad5* intron 1, *nad1* intron B/C and *nad7* intron 1) identified six different mitotypes (Supplementary Table S3), M1–M6 (Figure 1c; Supplementary Table S1) over all trees examined. In *P. sylvestris* var. *mongolica*, all populations except two were fixed for M1 and the overall frequency of M1 in the species was 99.2%. The two other mitotypes found in this species occurred in single individuals within different populations, M5 in population 22 and M6 in population 23. In *P. densiflora*, a strong geographical pattern for mtDNA variation was evident. Northern populations of this species (populations 24–27) were fixed for mitotype M3 (overall frequency in species equalled 34.5%), while southern populations (32–41) were fixed for mitotype M2 (overall frequency in species equalled 58.8%). In contrast, central populations sampled from Jilin province (populations 28–31) were fixed for either M2 (population 30) or M3 (population 28), or were polymorphic for M2 and M4 (populations 29 and 31). Mitotypes M3 and M4, which differed from M2 by a single-nucleotide substitution, occurred at very high frequency in *P. takahasii* and *P. funebris*, respectively. None of the mitotypes found in *P. sylvestris* var. *mongolica* (M1, M5 and M6) was recorded in either putative hybrid species.

The mitotype network (Figure 1d) indicated that mitotypes found in *P. sylvestris* var. *mongolica* formed one group while those present in the two putative hybrid species and *P. densiflora* formed another group. Total mtDNA diversity was much higher in *P. densiflora* ($H_T=0.522$) than in *P. sylvestris* var. *mongolica* ($H_T=0.028$) and *P. takahasii* ($H_T=0.020$), while average within-population diversity was similar ($H_S=0.028$ in *P. sylvestris* var. *mongolica*, $H_S=0.046$ in *P. densiflora*, and $H_S=0.029$ in *P. takahasii*). In *P. densiflora*, N_{ST} (0.919) was not significantly greater than G_{ST} (0.913) ($P>0.05$, Supplementary Table S4). As expected, analysis of molecular variance showed that most variation was distributed between species (97.55%) while variation between populations within species was significant only in *P. densiflora* (Supplementary Table S5).

Nuclear loci

The amount of nucleotide polymorphism in each species varied greatly among loci with the *dhn1* locus being most polymorphic in all four species (Supplementary Table S6). Average estimates of polymorphism over the eight loci were similar across species with silent polymorphism being three- to fivefold greater than polymorphism at nonsynonymous sites. *P. funebris* and *P. takahasii* exhibited similar levels of silent nucleotide diversity that were somewhat higher than those exhibited by *P. sylvestris* var. *mongolica* and *P. densiflora* (Table 2).

F_{ST} and net sequence divergence (Da) were calculated to measure genetic differentiation among species at each locus (Table 3). Results for Da were similar to F_{ST} and thus are not presented here. F_{ST} -values

were mostly significantly different from zero at each locus between *P. sylvestris* var. *mongolica* and one or other of the other three species. Significant F_{ST} -values were also detected at the *a3ip2*, *ccoamt* and *dhn1* loci between *P. densiflora* and *P. funebris*, at the *ccoamt*, *dhn1* and *erd3* loci between *P. densiflora* and *P. takahasii* and at the *pod* and *CesA2* loci between *P. funebris* and *P. takahasii*, respectively (Table 3). The genetic differentiation was much smaller between *P. densiflora* and *P. funebris* and *P. takahasii*, as compared with *P. sylvestris* var. *mongolica* and the latter two species.

We calculated Tajima's D , Fu and Li's D^* and F^* and Fay and Wu's H to detect departures from the standard neutral model of molecular evolution at each locus. Fu and Li's D^* tests showed a similar trend to F^* (data not shown). Most of the tests did not significantly deviate from the standard neutral model (Table 4). We detected significant negative D or H at the *ccoamt* locus in *P. sylvestris* var. *mongolica* and *P. densiflora*, and significant negative D and D^* at the *CesA2* locus in *P. sylvestris* var. *mongolica*. No significant value was detected in either putative hybrid species.

Haplotype genealogies constructed for each locus by NETWORK (Figure 2) could be grouped into three different types. One type exhibited by the *a3ip2* and *ccoamt* loci showed a high level of haplotype distinction between *P. sylvestris* var. *mongolica* and *P. densiflora*. Thus, 7 of the 12 haplotypes detected at the *a3ip2* locus were specific to *P. sylvestris* var. *mongolica*, while 3 were specific to *P. densiflora*. Similarly, at the *ccoamt* locus, two of the 15 haplotypes detected were specific to *P. sylvestris* var. *mongolica* while 9 were specific to *P. densiflora*. At neither locus were any haplotypes shared between these two taxa. All haplotypes possessed by *P. sylvestris* var. *mongolica* were separated from those possessed by *P. densiflora* by at least five evolutionary steps (mutations) based on most parsimonious relationships. The putative hybrid species, *P. takahasii* and *P. funebris*, possessed a mixture of haplotypes specific to *P. sylvestris* var. *mongolica* and *P. densiflora* as well as some haplotypes (alleles) specific to themselves.

The second type of genealogy was observed for five loci (*pod*, *c3h-1*, *CesA2*, *dhn1* and *erd3*) where one central haplotype shared by *P. sylvestris* var. *mongolica*, *P. densiflora* and at least one of the two putative hybrid species was ancestral to other haplotypes. In this genealogy, both *P. takahasii* and *P. funebris* also shared a few derived haplotypes that were specific to either *P. sylvestris* var. *mongolica* or *P. densiflora*, as well as possessing a few haplotypes not found in either putative parent species. In the final class of haplotype network exhibited for the *aqua-MIP* locus, the most common haplotypes were mainly found in either *P. sylvestris* var. *mongolica* or *P. densiflora* and one or both putative hybrid species. The one exception to this was for H2, which was present in all four taxa. Once again, a few haplotypes were recorded in *P. takahasii* and *P. funebris* that were not present in either putative parent species. Despite the different types of genealogies noted across the eight loci, two common features were evident: (1) haplotypes specific to either *P. sylvestris* var. *mongolica* or *P. densiflora* were commonly shared by *P. takahasii* and *P. funebris*, and (2) some haplotypes were specific to the putative hybrid species (that is, 10 haplotypes were specific to *P. funebris* and 14 to *P. takahasii*).

We also used the Bayesian clustering algorithm implemented in the program STRUCTURE to detect genetic admixture of the two possible hybrid species. Model selection based on the ΔK criterion (Evanno *et al.*, 2005) supported $K=2$. Values of LnPD showed an increase with increasing K using the original method of Pritchard *et al.* (2000), although the increase was very low when $K\geq 6$. The STRUCTURE clustering results for $K=2-6$ are presented in Supplementary Figure

Table 2 Average nucleotide diversity across eight nuclear loci in each of the four pine species

Species	Total				Nonsynonymous sites				Silent sites				
	n	L	S	θ_w	π	L	S	θ_w	π	L	S	θ_w	π
<i>P. sylvestris</i> var. <i>mongolica</i>	28	5417	112	0.00627	0.00579	1931.35	4	0.00069	0.00172	3282.65	55	0.00552	0.00759
<i>P. densiflora</i>	28	5458	113	0.00642	0.00595	1939.11	5	0.00088	0.00225	3205.89	62	0.00641	0.00777
<i>P. funebris</i>	12	5410	104	0.0076	0.00753	1938.3	5	0.0011	0.002	3226.7	62	0.00793	0.01065
<i>P. takahasii</i>	10	5426	98	0.0075	0.00808	1939	15	0.00302	0.00325	3257	85	0.01024	0.01075

Abbreviations: n, sample size; L, length in base pairs; S, number of segregating sites; θ_w , Watterson's parameter; π , nucleotide diversity. All indels were excluded from the analysis.

Table 3 Genetic differentiation at eight nuclear loci among (a) *P. sylvestris* var. *mongolica*, (b) *P. densiflora*, (c) *P. funebris* and (d) *P. takahasii*

Group	a vs b		a vs c		a vs d		b vs c		b vs d		c vs d	
	F_{ST}	D_a	F_{ST}	D_a	F_{ST}	D_a	F_{ST}	D_a	F_{ST}	D_a	F_{ST}	D_a
<i>a3ip2</i>	0.8052***	0.0085	0.5190***	0.0032	0.5963***	0.0043	0.3534*	0.0013	0.1876	0.0004	0.0650	-0.00002
<i>Ccoaomt</i>	0.8875***	0.0084	0.7292***	0.0048	0.7598***	0.0060	0.2067**	0.0003	0.1932*	0.0005	0.0154	-0.0004
<i>pod</i>	0.3849*	0.0065	0.0643	0.0004	0.3687*	0.0070	0.1665	0.0025	0.0122	0.0021	0.2757*	0.0057
<i>c3h-1</i>	0.1638*	0.0005	0.1262	0.0008	0.2045	0.0011	0.1615	0.0009	0.1658	0.0007	-0.1328	-0.0001
<i>CesA2</i>	0.3865*	0.0014	0.4167***	0.0005	0.4248*	0.0011	0.1850	0.0004	-0.1698	-0.0002	0.2482*	0.0003
<i>dhn1</i>	0.1981*	0.0024	-0.0334	-0.00005	0.3582*	0.0060	0.1703*	0.0010	0.2801*	0.0031	0.2515	0.0036
<i>erd3</i>	0.4659***	0.0010	0.4736*	0.0016	0.3676**	0.0011	0.1690	0.0003	0.2400*	0.0005	0.0437	0.00007
<i>aqua-MIP</i>	0.3886**	0.0038	0.0723	0.0017	0.1911	0.0021	-0.0197	-0.0002	0.0261	-0.0003	-0.1373	-0.0009
Average	0.4601	0.0041	0.2960	0.0016	0.4089	0.0036	0.1741	0.0008	0.1169	0.0009	0.0787	0.0010

Significant level: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Table 4 Neutrality test at each nuclear locus as measured by Tajima's D , Fu and Li's D^* and Fay and Wu's H .

Locus	<i>P. sylvestris</i> var. <i>mongolica</i>			<i>P. densiflora</i>			<i>P. funebris</i>			<i>P. takahasii</i>		
	D	D^*	H	D	D^*	H	D	D^*	H	D	D^*	H
<i>a3ip2</i>	0.4892	0.8907	-3.0154	NA	NA	NA	0.3046	-0.3973	0.8889	0.1350	0.8913	-1.5714
<i>ccoaoomt</i>	-1.8493*	-2.2413	-1.4892	-1.4089	-1.3125	-2.2530*	-0.2238	0.1597	-1.7818	0.1728	0.1348	-0.6389
<i>pod</i>	0.8708	-0.1036	1.0145	-0.5533	-0.5293	-4.5735	1.6709	1.1179	1.5000	0.6816	0.8913	0.2143
<i>c3h-1</i>	-0.2331	-0.5206	0.4558	-1.2429	-0.4453	-2.0733	0.8317	0.5428	0.4546	1.3299	0.8209	0.9286
<i>CesA2</i>	-2.1312*	-3.1453**	-1.2667	0.1208	-0.5416	-0.1477	0.8233	0.2314	0.9455	0.7944	0.2314	-0.0833
<i>dhn1</i>	-0.2325	-0.7284	3.2213	-0.0336	-0.0672	7.3464	-1.0356	-1.0030	4.2381	0.2274	0.3269	2.1429
<i>erd3</i>	-0.0503	0.1405	-2.9930	-0.8440	-0.7560	-2.3020	0.1591	-0.4623	0.7091	0.2626	0.0638	1.0667
<i>aqua-MIP</i>	-0.4165	-1.1908	1.1923	-0.2909	0.1537	1.9368	-0.0452	0.5717	3.6727	-0.6265	-0.6392	2.5714

Significant level: * $P < 0.05$. NA: failed to be computed for lack of enough variation. All indels were excluded from the analysis.

Table 5 Results of demographic analysis based on chlorotypes performed using mismatch distribution, neutrality tests and LAMARC

Group	θ_0	θ_1	SSD (P-value)	RAG (P-value)	Fu's F_S	Tajima's D	Growth rate (g)
<i>P. sylvestris</i> var. <i>mongolica</i> (I)	0	99 999	0.078 (0.112)	0.277 (0.210)	-9.448***	-1.837**	888.6
<i>P. densiflora</i> (II)	0.466	39.766	0.045 (0.276)	0.145 (0.411)	-3.572 (0.063)	-1.374 (0.063)	542.4
<i>P. densiflora</i> (species-specific) (III)	0.002	99 999	0.122 (0.183)	0.500 (0.535)	-3.849**	-1.347*	773.2

Abbreviations: RAG, the Harpending's Raggedness index; SSD, sum of squared deviations. θ_0 and θ_1 are pre-expansion and post-expansion population sizes. Significant level: ** $P < 0.01$ and *** $P < 0.001$.

S2). When $K=2$, both *P. funebris* and *P. takahasii* were shown to comprise a genetic mixture of *P. densiflora* and *P. sylvestris* var. *mongolica*, with more of their ancestry coming from the former species.

Coalescent-based estimates from LAMARC indicated that the input of polymorphism to the assumed hybrid species from their putative

parental species following historical migration was very high relative to the mutation rate ($M=m/\mu$). The most probable estimates of M ranged from 158 to 619, with the highest migration estimated from *P. densiflora* into *P. funebris* and *P. takahasii* ($M=619-573$) (Supplementary Table S7).

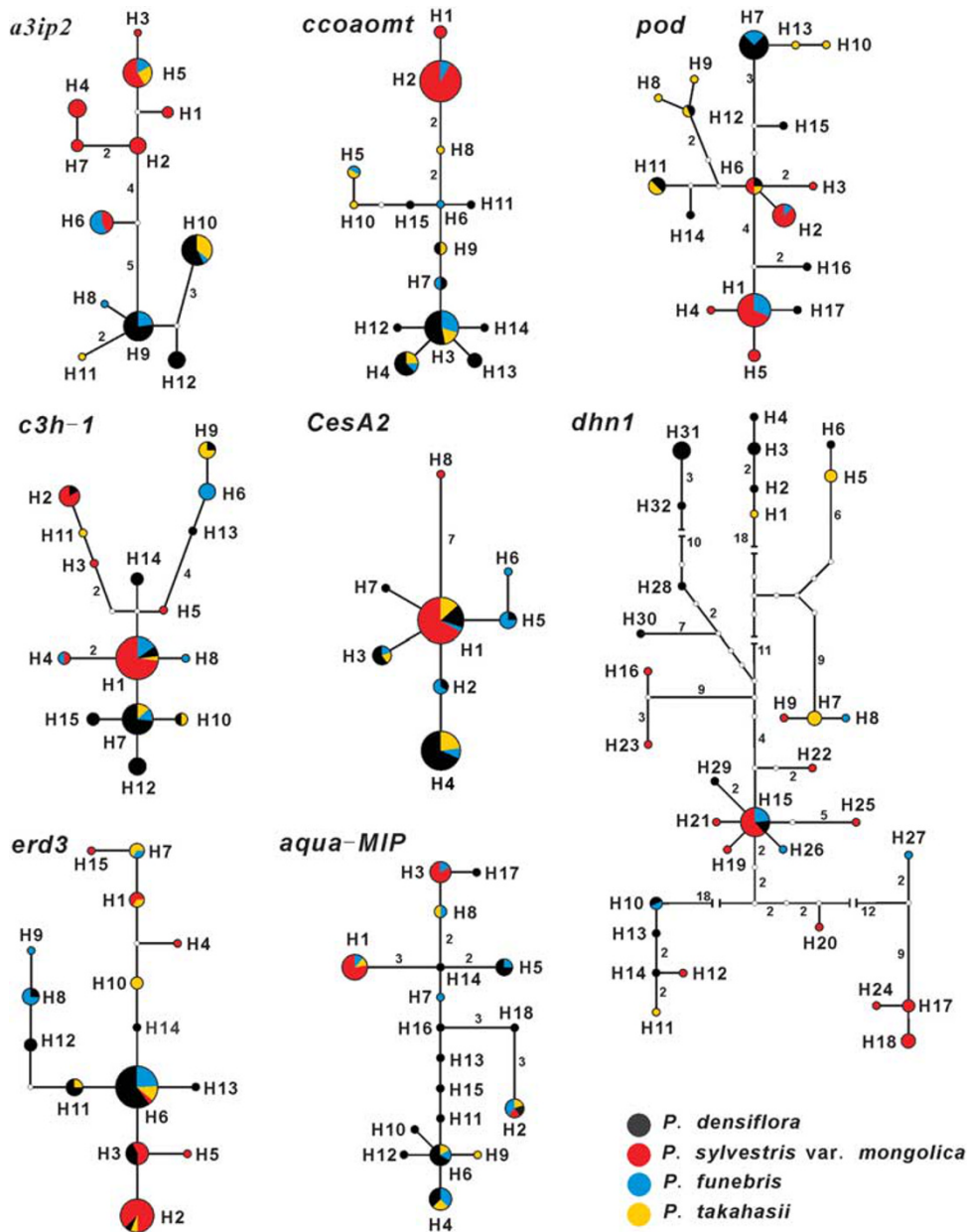


Figure 2 Haplotype genealogies for eight nuclear loci. The color of each sector of a circle indicates the frequency of a haplotype recorded in each pine species. The size of a circle is proportional to the frequency of a haplotype across the four pines. Branch lengths longer than one mutation step are marked on each branch.

Demographic expansion analysis based on cpDNA sequences

The mismatch distribution for all chlorotypes recorded in *P. sylvestris* var. *mongolica* (group I) showed a unimodal pattern (Figure 3a), which is indicative of a past range expansion in this species. In *P. densiflora* (group II), two peaks were observed in the mismatch distribution (Figure 3b), which might reflect introgression of some chlorotypes from *P. sylvestris* var. *mongolica* to *P. densiflora*. This possibility is supported by the fact that a unimodal mismatch distribution was obtained when chlorotypes shared with *P. sylvestris* were excluded from analysis (group III, Figure 3c). Thus, a past range expansion might also be inferred for *P. densiflora*. Further analyses of the variance (SSD) and raggedness index suggested that the curves did not differ significantly from those of distributions expected from a model of sudden population expansion (Table 5).

Sudden and recent range expansions of both *P. sylvestris* var. *mongolica* and *P. densiflora* in Northeast China are also supported by significant negative values obtained for F_S and Tajima's D values in chlorotype groups I and III, and by large values obtained for the exponential population growth rate parameter ' ρ ' derived by LAMARC tests (Kuhner, 2006) on all three groups of cpDNA sequences, groups I–III (Table 5).

Favored scenarios for the origins of *P. funebris* and *P. takahasii* based on ABC simulations

Posterior probabilities clearly favored the admixture origin hypothesis for both *P. funebris* and *P. takahasii* (Table 6, Supplementary Figure S1, 3, Supplementary Tables S8, S9, S10). More specifically, our ABC simulations that included models of changes in effective population

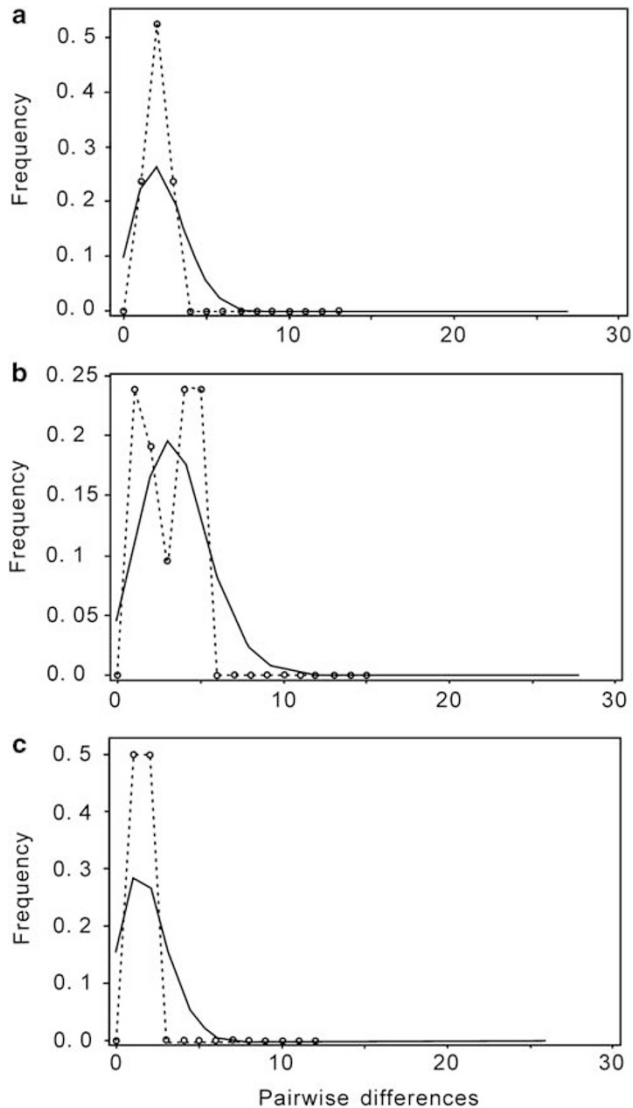


Figure 3 Results of the mismatch distribution analysis for (a) all chlorotypes recorded in *P. sylvestris* var. *mongolica*, (b) all chlorotypes recorded in *P. densiflora* and (c) chlorotypes specific to *P. densiflora*. Continuous lines show the distributions expected for an expanding population, while the dotted lines represent the observed distributions of pairwise differences among samples.

size supported the hypothesis that *P. sylvestris* var. *mongolica* and *P. densiflora* expanded in the past. *P. sylvestris* var. *mongolica* was estimated to expand around 190 (95% CI: 68–248) thousand years ago (kya) and *P. densiflora* about 110 (95% CI: 23–220) kya. In addition, the posterior parameter estimations suggested that the admixture origins of *P. funebris* and *P. takahasii* from *P. sylvestris* var. *mongolica* and *P. densiflora* occurred around 33 (median, 95% CI: 2–73) kya and 35 (median, 95% CI: 2–73) kya, respectively. The two parental species were estimated to have diverged from their most recent common ancestor (MRCA) around 1.85 million years ago.

DISCUSSION

Our comparison of maternally inherited mtDNA, paternally inherited cpDNA and biparentally inherited nuclear DNA sequence variation within and between four closely related pine species in Northeast

Table 6 Description of all scenarios used in the approximate Bayesian computation analysis in DIYABC 1.0.4.39 to test the origin of the two putative hybrid species, respectively

Step	Scenario	Posterior probability	Credibility interval
Origin of <i>P. funebris</i>	1.1	0.95978	(0.94977, 0.96978)
	1.2	0.00000	(0.00000, 0.00000)
	1.3	0.00132	(0.00085, 0.00177)
	1.4	0.03877	(0.02882, 0.04871)
	1.5	0.00000	(0.00000, 0.00000)
	1.6	0.00013	(0.00009, 0.00019)
Origin of <i>P. takahasii</i>	2.1	0.89481	(0.87701, 0.91257)
	2.2	0.00000	(0.00000, 0.00000)
	2.3	0.03691	(0.02774, 0.04610)
	2.4	0.06413	(0.05002, 0.07826)
	2.5	0.00000	(0.00000, 0.00000)
	2.6	0.00416	(0.00263, 0.00568)

Each contained six scenarios with three of them under population size change models (scenario 1–3) or not (scenario 4–6) (see Supplementary Figure S1 and 3). The relative posterior probabilities and 95% confidence intervals for each scenario were computed via the logistic regression on 1% of the closest data sets to the observed data.

China, revealed that the two widespread species, *P. sylvestris* var. *mongolica* and *P. densiflora*, were genetically diverged and delimited by mtDNA, cpDNA and two of eight nuclear genes examined. In areas of sympatry, some hybrids between these two species were identified based on chlorotype and nuclear genotype composition. Population genetic analysis suggested that both species had experienced rapid range expansion during the late Quaternary. This we propose led to secondary contact between them and subsequent interspecific hybridization, which is possibly resulted in the homoploid hybrid origin of the narrowly distributed pines, *P. funebris* and *P. takahasii*, each of which possesses a mixture of cpDNA and nuclear haplotypes that distinguish *P. sylvestris* var. *mongolica* from *P. densiflora*.

Genetic divergences between *P. densiflora* and *P. sylvestris* var. *mongolica*

Closely related species are often divergent at some loci, but share alleles at other loci (Nosil *et al.*, 2009). Sharing of alleles may result from incomplete lineage sorting and/or interspecific introgression (Gow *et al.*, 2006). *P. densiflora* and *P. sylvestris* var. *mongolica* differed in mitotype composition (Figure 1; Supplementary Table S1) with *P. densiflora* containing three closely related mitotypes (M2, M3 and M4), while *P. sylvestris* contained the distantly related M1 mitotype at very high frequency plus two very rare mitotypes, M5 and M6. According to the ‘four gametic criterion’, mitotypes M5 and M6 were assumed to be recombinants of M1 and M2 (Hudson and Kaplan, 1985; Jaramillo-Correa and Bousquet, 2005), which is consistent with their distribution only in areas of sympatry with *P. densiflora*.

P. densiflora and *P. sylvestris* var. *mongolica* were also largely distinguished by the chlorotypes they possessed with chlorotypes C8 and C1 occurring at high frequency in *P. densiflora* (88.5%) and *P. sylvestris* (94.5%), respectively. These two chlorotypes were separated by two steps in a chlorotype network in which they were located centrally (Figure 1). The remaining 11 chlorotypes identified in the analysis were rare and according to the network were derived from one or other of the two common chlorotypes. Three chlorotypes (C1, C2 and C5) found in *P. sylvestris* var. *mongolica* were present at low frequency in sympatric populations of *P. densiflora*, indicating recent cryptic introgression.

Taken together, our analysis of mtDNA and cpDNA variation showed that the two widespread species investigated, *P. densiflora* and *P. sylvestris* var. *mongolica*, can be largely delimited by both the mtDNA and cpDNA haplotypes they possess, although a low level of chlorotype sharing occurs in areas of sympatry. The finding for mtDNA contrasts with those of some previous surveys of variation in coniferous species, which showed a high degree of mitotype sharing across closely related species and an inability to delimit species according to mitotype (Du *et al.*, 2009; Zhou *et al.*, 2010).

The two widespread pine species were also distinguished by different nuclear haplotypes at two of eight nuclear loci surveyed (*a3ip2* and *ccoamt*), and F_{ST} values (Table 3) were significant at these loci; however, at the remaining six loci there was considerable haplotype sharing between the species (Figure 2). Significant negative Tajima's D or Fay and Wu's H were only observed at the *ccoamt* locus in *P. sylvestris* var. *mongolica* and *P. densiflora*, indicating a higher signature of selection or demographic expansion at this locus. The *a3ip2* gene encodes a protein that interacts with the protein ABI3 that possibly functions in the control of bud growth and flowering (Palmé *et al.*, 2009), while the *ccoamt* gene encodes a key enzyme for lignin biosynthesis involved in plant defense (Almagro *et al.*, 2009). Allelic substitutions at both genes may have had important roles in the adaptive divergence and reproductive isolations of the two species. Thus, complete lineage sorting at these two loci might be due to their linkage to speciation genes (hitchhiking) or to their direct role in adaptive divergence. If this is correct, the two genes would be expected to have been subjected to strong selection-driven lineage sorting during speciation and also following any subsequent introgression events (Via, 2009). The shared haplotypes (alleles) at the remaining six nuclear loci examined, which were located at central positions in the haplotype networks (Figure 2), may derive from ancestral polymorphisms, whereas those positioned at the tips of branches are more likely due to introgression between the two species (Zheng and Ge, 2010). If these genes are not linked to speciation genes or have little direct role in the adaptive divergence of the two species, lineage sorting of ancestral polymorphisms and re-sorting of introgressed alleles at these loci would be expected to occur more slowly (Ting *et al.*, 2000) and therefore explain the haplotype sharing observed.

Glacial refugia and range expansions of *P. densiflora* and *P. sylvestris*

The three mitotypes recorded within *P. densiflora* differed in their geographical distribution although N_{ST} was not significantly greater than G_{ST} (Supplementary Table S6). Whereas mitotype M2 was widely distributed in the southern part of Northeast China, M3 was fixed in multiple populations sampled from the Changbai Mountains to the north. This geographical pattern of variation suggests that the species' range may have been fragmented in the past into at least two isolated glacial refugia located in the northeast and south (Supplementary Figure S4a), respectively. This is of interest as studies of other forest trees have also indicated the occurrence of more than one glacial refugium for forests in northeast Asia (for example, Aizawa *et al.*, 2007; Bai *et al.*, 2010).

In contrast to the situation for mitotype variation, all populations of *P. densiflora* shared a common chlorotype (C8). Such uniformity could have been caused by the homogenizing effect of a high level of gene flow through long-distance dispersed pollen preventing cpDNA divergence occurring among populations in geographically isolated glacial refugia (Supplementary Figure S4a). The occurrence of a 'star-like' phylogeny of rare chlorotypes derived from chlorotype C8 (Figure 1) indicates that an extensive expansion in the geographical

range of *P. densiflora* occurred in the recent past. Similarly, the presence of one widespread mitotype in *P. sylvestris* var. *mongolica* together with a 'star-like' phylogeny of rare chlorotypes derived from the most common chlorotype (C1) suggests an extensive range expansion of this species in Northeast China in the relatively recent past (Supplementary Figure S4b). The latter is consistent with the findings of a recent phylogeographic study of *P. sylvestris* over its Eurasian distribution, which showed that central Asia was one of its glacial refugia from where the species colonized Northeast China recently and expanded there (Naydenov *et al.*, 2007).

The occurrence of recent range expansions of *P. densiflora* and *P. sylvestris* var. *mongolica* were also supported by the significant negative values obtained for Fu's F_S and Tajima's D (Table 5), the demonstration of unimodal mismatch distributions, and the large population expansion rates ($g > 500$; Table 5) based on cpDNA sequence data, plus the effective population size change models estimated by ABC based on eight nuclear loci. Neutrality tests of nuclear data partly supported a recent expansion hypothesis for both species in that the estimated values of Tajima's D and Fu and Li's D^* were mostly negative; however, because of limited sample size, departures from the standard neutral model of molecular evolution were not statistically significant (Table 4). The range expansions of *P. sylvestris* var. *mongolica* and *P. densiflora* were estimated to have occurred about 190 and 110 kya, respectively, based on nuclear data (Supplementary Tables S9–10). These dates suggest that the Last Glacial Maximum (~20 kya) probably had little effect on the range contractions and expansions of these two species in the late Quaternary. Instead, the dates are more consistent with the period following the largest Quaternary glaciation recorded for the Asian mountains, which began approximately 800 kya and continued until 200 kya (Zheng *et al.*, 2002; Wu *et al.*, 2010).

It is feasible that during this glaciation, the range of *P. densiflora* was fragmented into different refugia in Northeast China, and following the end of this glacial period it expanded its range both southward and northeastward. At the same time, *P. sylvestris* is believed to have colonized Northeast China from central Asia (Naydenov *et al.*, 2007), and after expanding its range southward will have come into contact and hybridized with *P. densiflora* (Supplementary Figures S4b and c).

Hybrid origins of *P. funebris* and *P. takahasii*?

Evidence supporting the hypothesis that both *P. funebris* and *P. takahasii* are possibly homoploid hybrid species emerged from the analysis of cp, mt and nuclear DNA variation. Both species contained cp and nuclear haplotypes that distinguished the two putative parent species, *P. densiflora* and *P. sylvestris* var. *mongolica*, and STRUCTURE confirmed that they were a genetic mixture of the two widespread species but with a greater proportion of their nuclear ancestry coming from *P. densiflora* (Table 3, Supplementary Figure S3). This is consistent with the results of a previous study based on allozyme polymorphisms (Szmids and Wang, 1993). Moreover, the most favored scenarios that emerged from the ABC simulations conducted strongly supported an admixed origin of *P. funebris* and *P. takahasii* from the other two species approximately 33–35 kya. Both putative hybrid species were almost fixed for different mitotypes only found in *P. densiflora*, indicating that different individuals of *P. densiflora* had served as the maternal parents of *P. funebris* and *P. takahasii*, respectively.

These two putative hybrid species are likely to be reproductively isolated from their parents through spatial/habitat isolation. *P. funebris* grows at higher elevations relative to either parent in the Changbai Mountains, while *P. takahasii* occurs in wetter habitats around the

Xingkai Lake (Cheng and Fu, 1978) from which both putative parents are excluded. Such ecological differentiation from parental taxa appears very common for most recorded homoploid hybrid species (Gross and Rieseberg, 2005). For example, in pines, *P. densata*, which is derived from hybridization between *P. yunnanensis* and *P. tabulaeformis*, inhabits a high mountain environment where the parental species are apparently unable to grow (Wang *et al.*, 2001). It has also been reported that whereas *P. densiflora* sheds its pollen in April, both putative hybrid species and *P. sylvestris* var. *mongolica* release their pollen from May to June (Cheng and Fu, 1978). This difference in pollen shedding time might therefore also contribute to reproductive isolation between the two putative hybrid species and *P. densiflora*, although this possibility will need to be verified by detailed analysis.

Although one scenario for the origin of *P. funebris* and *P. takahasii* is homoploid hybrid speciation, an alternative scenario that cannot be discounted is that both taxa originated by divergence (for example, from *P. densiflora*), possibly as a result of adaptation to different habitats, and subsequently were introgressed (for example, by *P. sylvestris* var. *mongolica*) (see Wachowiak *et al.*, 2011, for a possible example of divergence followed by introgression in other pine species). In this case, their hybrid nature might be coincidental and not directly responsible for their origin, in that the introgressed genes may have had no part in their reproductive isolation from their parents. Deciding between these two evolutionary scenarios, that is, homoploid hybrid speciation or divergence from a parent species followed by introgression, is difficult. If in future it is shown that genes contributed by both parent species were instrumental in causing the reproductive isolation of *P. funebris* and *P. takahasii*, for example by enabling a shift in habitat, then divergence following hybridization might be assumed and thus both species could be concluded to be homoploid hybrid species. Until then, however, we should continue to consider *P. funebris* and *P. takahasii* as putative hybrid species rather than as fully proven hybrid species.

The finding that only *P. densiflora* served as the maternal parent of both putative hybrid species implies mainly unidirectional hybridization between *P. sylvestris* var. *mongolica* and *P. densiflora* in the wild as reported to have occurred between some other pine species (Watano *et al.*, 2004; Liston *et al.*, 2007; Wachowiak and Prus-Głowacki, 2008). Such unidirectional hybridization might have two alternative causes. First, it is feasible that *P. densiflora* stops shedding pollen before the ovules of *P. sylvestris* var. *mongolica* become receptive, whereas the later developed ovules of *P. densiflora* may be available to early-shed pollen of *P. sylvestris*. Second, hybrid survival may be species-biased (Currat *et al.*, 2008) with only those having *P. densiflora* as a maternal parent being viable. This has been reported to be the case for some other hybrids between other pine species (Wachowiak *et al.*, 2005), that is, hybrids mothered by one species are viable, but are inviable if produced by the reciprocal cross.

CONCLUSION

Our genetic analysis has shown that *P. funebris* and *P. takahasii* contain highly diverged chlorotypes and nuclear alleles that distinguish *P. sylvestris* var. *mongolica* and *P. densiflora*. During the most severe glacial stage of the Quaternary, *P. sylvestris* and *P. densiflora* probably remained allopatric: the former species surviving in glacial refugia in central Asia and Europe (Naydenov *et al.*, 2007) while the latter was restricted to two or more refugia in Northeast China. At the end of this period, *P. sylvestris* var. *mongolica* is thought to have colonized Northeast China and expanded its range southward into the distribution range of *P. densiflora*, which had expanded its range north-eastward. Such contact lead to interspecific hybridization and the

possible origin of two putative homoploid hybrid species, *P. funebris* and *P. takahasii*. Although our findings based on detailed analysis of large population genetic data sets are consistent with the homoploid hybrid origin of these two species, we are unable to exclude fully other possible origins for them. For example, an origin by divergence from *P. densiflora* followed by introgression from *P. sylvestris* var. *mongolica*. This emphasizes the difficulty of confirming homoploid hybrid speciation from such data sets and the need to study where possible the process 'in action' to fully understand the mechanisms that drive it (Abbott *et al.*, 2010).

DATA ARCHIVING

Data have been deposited at Dryad: doi:10.5061/dryad.cb052n9j.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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